Development of real-time reverse transcriptase PCR assays for the detection of Punta Toro virus and Pichinde virus.

Christopher P. Stefan
christopher.p.stefan.ctr@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America
Kitty Chase
catherine.j.chase.ctr@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America
Susan Coyne
susan.r.coyne.civ@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America
David A. Kulesh
david.a.kulesh.civ@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America
Jeffrey W. Koehler
jeff.w.koehler.ctr@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America
Timothy D. Minogue (corresponding author)
timothy.d.minogue.civ@mail.mil
Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, United States of America

Abstract

Background: Research with high biocontainment pathogens such as Rift Valley fever virus (RVFV) and Lassa virus (LASV) is expensive, potentially hazardous, and limited to select institutions. Surrogate pathogens such as Punta Toro virus (PTV) for RVFV infection and Pichinde virus (PICV) for LASV infection allow research to be performed under more permissive BSL-2 conditions. Although used as infection models, PTV and PICV have no standard real-time RT-PCR assays to detect and quantify pathogenesis. PTV is also a human pathogen, making a standardized detection assay essential for biosurveillance. Here, we developed and characterized two real-time RT-PCR assays for PICV and PTV by optimizing assay conditions and measuring the limit of detection (LOD) and performance in multiple clinical matrices.

Methods: Total nucleic acid from virus-infected Vero E6 cells was used to optimize TaqMan-minor groove binder (MGB) real-time RT-PCR assays. A 10-fold dilution series of nucleic acid was used to perform analytical experiments with 60 replicates used to confirm assay limits of detection (LODs). Serum and whole blood spiked with 10-fold dilutions of PTV and PICV virus were assessed as matrices in a mock clinical context. The second derivative method with the Roche LightCycler 480 software version 1.5.1 was used to determine Cq.
Results: Optimized PTV and PICV assays had LODs of 1000 pfu/ml and 100 pfu/ml, respectively, and this LOD was confirmed in 60/60 (PTV) and 58/60 (PICV) positive replicates. Preliminary mock clinical LODs remained consistent in serum and whole blood for PTV and PICV at 1000 pfu/ml and 100 pfu/ml. An exclusivity panel showed no cross reaction with near neighbors.
Conclusions: PTV and PICV Taq-man MGB based real-time RT-PCR assays developed here showed relevant sensitivity and reproducibility in samples extracted from a variety of clinical matrices. These assays will be useful as a standard by researchers for future experiments utilizing PTV and PICV as infection models, offering the ability to track infection and viral replication kinetics during research studies.

Background

Both Rift Valley fever virus (RVFV) and Lassa fever virus (LASV) are highly pathogenic viruses endemic to Africa. RVFV, within the *Bunyaviridae* family, is a mosquito-borne, biosafety level (BSL)-3 select agent of major public health and economic concern, affecting humans and livestock throughout Africa (Adam et al., 2009; Daubney et al., 1931; Digoutte and Peters, 1989; Durand et al., 2003; Madani et al., 2003; Meegan et al., 1979) and the Arabian Peninsula (Shoemaker et al., 2002). LASV, within the *Arenaviridae* family, is a BSL-4 select agent responsible for approximately 500,000 infections yearly in West Africa (Birmingham and Kenyon, 2001; Buckley et al., 1970). Both of these viruses can result in a hemorrhagic fever syndrome and can cause large outbreaks in endemic regions.

Research with either of these pathogens is hazardous, expensive, and limited to studies at select institutions by approved individuals. As such, BSL-2 infection models for both viruses have been developed: Punta Toro virus (PTV) for RVFV infection and Pichinde virus (PICV) for LASV infection. While these models have been used for multiple pathogenesis and therapeutics studies (Anderson et al., 1990; Buchmeier and Rawls, 1977; Fisher et al., 2003; Gowen et al., 2005; Gowen et al., 2006a; Gowen et al., 2006c; Jahrling et al., 1981; Lucia et al., 1989; Perrone et al., 2007; Smee et al., 1993), there are no real-time PCR assays described in the literature for these viruses. The availability of well characterized assays to monitor viral replication kinetics would aid these research efforts.

PTV infection in mice (Gowen et al., 2006a) and hamsters (Anderson et al., 1990; Fisher et al., 2003; Perrone et al., 2007) results in disease similar to RVFV infection in humans and is an established BSL-2 surrogate infection model for RVFV. PTV, a mosquito-transmitted bunyavirus, typically causes a mild and self-limiting infection in humans but may progress to an acute, febrile illness (Bartelloni and Tesh, 1976). Both RVFV and PTV consist of three RNA segments: the L, S, and M segments (Lihoradova et al., 2013; Xu etal., 2007). The L segment encodes the viral polymerase, the S segment contains the nucleoprotein and the nonstructural protein NSs, and the M segment encodes the two glycoproteins Gn and Gc as well as the nonstructural protein NSm.

PICV causes a similar disease in hamsters (Buchmeier and Rawls, 1977; Gowen et al., 2005; Gowen et al., 2006c; Smee et al., 1993) and guinea pigs (Jahrling et al., 1981; Lucia et al., 1989) as LASV infection in humans. Both PICV and LASV are arenaviruses with a genome comprised of the L and S RNA segments (Liang et al., 2010; Lukashevich 2013). The L segment encodes the viral polymerase and the Z protein, and the S segment encodes the nucleoprotein and the glycoprotein precursor GPC which is cleaved to yield the glycoproteins GP1 and GP2.

In this study, we designed two TaqMan-based real-time RT-PCR assays for detection of PTV and PICV. These assays were characterized and evaluated using cell culture supernatant from PTV or PICV infected cells and mock clinical samples. Overall, these assays could benefit the scientific community using animal models as surrogates for RVFV and LASV infection as well as biosurveillance for PTV infections in humans.

Methods

Viruses and cells. The Adames strain of PTV and the CO AN 4763 strain of PICV were provided by Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, Galveston, TX). Each virus was initially passaged in Vero E6 cells to generate stock virus from the cell culture supernatant. Virus stock titers were determined by standard plaque assay using 0.6% (w/v) SeaKem ME agarose (Lonza, Basel, Switzerland) and a secondary overlay containing 5% neutral red (Life Technologies, Grand Isle, NY). Vero E6 cells were maintained in complete Eagle's Minimum Essential Medium (cEMEM, Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin G (Life Technologies), and 100 mg/ml streptomycin (Life Technologies). Cells were incubated at 37°C with 5% CO_2. RNA from the cell culture supernatant was purified using Trizol LS (Life Technologies) and the Qiagen EZ1 robot with the EZ1 Virus Mini Kit (Qiagen, Valencia, Ca) according the manufacturer's directions.

Real-time RT-PCR assay design and downselection. Primers and TaqMan-minor groove binder (MGB) probe pairs were designed for PICV and PTV using Primer Express version 2.0 (Applied

Biosystems, Foster City, CA) and AlleleID 7.73 (PREMIER Biosoft, Palo Alto, CA). Primer/probe pairs (see Supplementary Table 1) were designed for PICV (L segment, GenBank# JN378748; S segment, GenBank# JN378747) and PTV (S segment, GenBank# EF201835; M segment, GenBank# DQ363407.1; L segment, GenBank# DQ363408.1). Primers and probe were ordered from Life Technologies.

Initial primer down selection was accomplished by testing for amplicon formation using purified nucleic acid from virus-infected cell culture supernatant and SYBR Green (Life Technologies), diluted according to manufacturer's protocol. Resultant amplicons were run on an ethidium bromide gel; primer pairs were selected based on a single, clean PCR product of the correct size. Downselected primer pairs were then evaluated using with the appropriate TaqMan-MGB probe and the Invitrogen SuperScript One-Step RT-PCR Kit (Life Technologies) with added bovine serum albumin (BSA) 20 mg/ml (Sigma, St. Louis, MO). Assays were run on the Roche LightCycler 2.0 (Roche Applied Science, Indianapolis, IN) or the LightCycler 480 (Roche) with a final concentration of 4 mM $MgSO_4$ and 0.25 mg/ml BSA with the following cycling conditions: 50^{o}C for 15 min (1 cycle); 95^{o}C for 5 min (1 cycle); 95^{o}C for 1 sec and 60^{o}C for 20 sec (45 cycles); and 40^{o}C for 30 sec (1 cycle). A single fluorescence read was taken at the end of each 60^{o}C step, and a sample was considered positive if the Cq value was less than 40 cycles.

Sensitivity testing was conducted for each assay using total nucleic acid isolated from cell culture supernatants from PICV and PTV infected Vero E6 cells. These supernatants were previously titered by plaque assay, so the limit of detection (LOD) was determined based on the number of pfu/ml. Purified RNA was serially diluted 10 fold into water, and 5 µl of the diluted RNA was run with each assay in triplicate. The preliminary LOD was determined based on 3/3 replicates being positive (<40 Cq), and 60 replicates at this preliminary LOD was conducted for LOD confirmation.

Mock Clinical LOD determination. Preliminary LODs for PTV or PICV in water, serum, and whole blood (BioreclamationIVT, Baltimore, MD) were determined by serially diluting known concentrations of virus into matrix in triplicate. RNA at each dilution was extracted using TRIzol LS and the EZ1 (Qiagen) according to the manufacturer's instructions, and real-time RT-PCR was performed on the extracted RNA with SuperScript One-Step RT-PCR Kit as described previously.

Exclusivity/Inclusivity panel. PTV and PICV probes and primers were tested against a panel of extracted viral nucleic acid samples. West Nile virus (UCC# Flavi022) and dengue virus serotypes 1-4 (UCC# Flavi029, UCC# Flavi030, UCC# Flavi031, and UCC# Flavi032) were provided by the Unified Culture Collection (UCC) maintained at USAMRIID. Rift Valley fever virus, Lassa fever virus (strains Josiah, Weller, and Pinneo), Mozambique virus, Junín virus, Machupo virus (Carvalo), Mobala virus, and Heartland virus are all maintained at USAMRIID.

Real-time PCR was performed with SuperScript One-Step RT-PCR Kit as described before. PTV and PICV were used as positive controls.

Statistical analysis. Cq values were calculated using the second derivative method with the Roche LightCycler 480 software version 1.5.1. GraphPad Prism v. 6.04 graphing software (GraphPad, La Jolla, CA) was used to plot sample data.

Results and Discussion

Assay Design/Optimization

Initial evaluations identified optimal primer and probe concentrations for each assay combination, and preliminary downselection testing identified a final assay for each virus (Table 1). The assays target the highly conserved polymerase genes, and empirical testing determined an optimal annealing temperature of 60°C with optimal primer and probe concentrations of 0.5 and 0.2 μM, respectively. Sanger sequencing confirmed the amplicon as virus-specific (Table 1).

Analytical LOD determination.

To have confidence in assay sensitivity, we conducted analytical evaluations including a preliminary LOD and a confirmation of LOD using nucleic acid extracted from virus-infected Vero E6 cell culture supernatant. Testing of eight, 10-fold serial dilutions from each virus ranging from 10^6 to 10^{-1} pfu/ml with each assay identified the preliminary LOD in which all three replicates were positive (Figure 1a). This testing showed the preliminary LODs were 1,000 pfu/ml and 100 pfu/ml for the PTV and PICV assay, respectively. In each case, we confirmed the preliminary LOD in a statistically robust manner (Figure 1b) using 60 replicates at the preliminary LOD. For PTV, all 60 replicates fell below the cutoff with an average Cq of 37.73, and 58/60 replicates were positive for the PICV assay with an average Cq of 36.16. The coefficient of variation for PTV and PICV was 1.15% and 2.13%, respectively. Exclusivity testing of primers and probes against a panel of extracted viral RNA were all negative.

Mock Clinical LOD determination.

Inhibitors of PCR are found in complex matrices such as blood (Akane et al., 1994) or stool (Monteiro et al., 1997; Widjojoatmodjo et al., 1992), and these inhibitors can carry through RNA preparation methods, affecting real-time RT-PCR results and impacting assay sensitivity (Kramvis et al., 1996). To further characterize these assays, we spiked each virus into water, human sera, and whole blood and performed six, 10-fold serial dilutions with matrix followed by nucleic acid extraction to re-establish preliminary LODs. Final concentrations ranged from 10^6 to 10^1 pfu/ml. For each matrix tested, water, serum, or whole blood, the LOD for PTV remained consistent at 1,000 pfu/ml as determined by 3/3 replicates falling below the 40 Cq cutoff value (Figure 2a). Similarly for PICV, the LOD remained unchanged when compared to the analytical characterizations at 100 pfu/ml for all three matrices tested (Figure 2b). In both instances,

evaluation criteria dictated Cq values greater than 40 be considered negative and given a final value of 40. Overall, these evaluations showed little to no impact or inhibition due to clinical matrix carryover for these assays.

Conclusion

Both RVFV and LASV are highly pathogenic viruses of significant public health and economic concern, and both are considered biothreat agents. Use of RVFV is limited to BSL-3 conditions, and LASV is restricted to the highest level of containment, BSL-4. Due to these restrictions and the limited access to these viruses, BSL-2 models for both viruses have been developed for pathogenesis and therapeutic studies. To date, the authors are unaware of published real-time RT-PCR assays for either PTV or PICV, so we developed and characterized real-time PCR assays for both PICV and PTV.

A series of primers and probes for each virus targeting the highly conserved polymerase gene were optimized and tested for assay LOD and performance in multiple clinical matrices. These assays will help track viral kinetics when utilizing PTV and PICV as infection models in animal models. Since PTV can also clinically infect humans, this assay can be used for biosurveillance studies or for diagnostics.

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Figure Legends and Tables

Table 1. Primers for PTV and PICV

Virus	Primers/probe	Sequence (5'-3')	Conc. (µM)	Amplicon
PICV	F3512	CATGTGTGGCCCCCATTT	0.5	63 bp
	R3574	TCAGTTGTTAGGCAAAGTGGTCTT	0.5	
	P3532S-MGB	6FAM-AATGGTCCATTGACACGG-MGBNFQ	0.2	
PTV	F430	CAGATAGCTGCTGCCATTTTACA	0.5	66 bp
	R495	GCTTTTAAGTTTCCCAGCCAAA	0.5	
	P454S-MGB	6FAM-CTCATTATTGTGGGCTCAT-MGBNFQ	0.2	

Figure 1. Analytical LOD for the PTV and PICV assays.

Preliminary and confirmatory LODs were performed with primer probe combinations listed in Table 1. A) Preliminary LODs were determined as having 3/3 replicate Cq values below 40 using serial 10-fold dilutions of PTV and PICV extracted RNA from water. Error bars represent

the standard deviation of three replicates. B) Confirmatory LODs were demonstrated with 60 replicates of PTV and PICV RNA extracted from water. Numbers in parenthesis represent the number of replicates with Cq values below the cutoff line. Each replicate is shown as an individual point with the bars representing the mean and standard deviation. In each assay, replicates that had no amplification curves or Cq values falling above 40 were given a base value of 40.

Figure 2. Mock clinical LODs for the PTV and PICV assays.
Total RNA extracted from 10-fold serial dilutions of (A) PTV and (B) PICV spiked into water, sera, and whole blood were extracted in triplicate and tested with the final PTV and PICV assay listed in Table 1. In each assay, replicates that had no amplification curves or Cq values falling above 40 were given a base value of 40. Error bars represent the standard deviation of three replicates.

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